

## DNA damage and mutation in human cells exposed to nitric oxide *in vitro*

T. NGUYEN\*, D. BRUNSON\*, C. L. CRESPI†, B. W. PENMAN†, J. S. WISHNOK\*, AND S. R. TANNENBAUM\*‡

\*Department of Chemistry and Division of Toxicology, Massachusetts Institute of Technology, 77 Massachusetts Avenue, 56-311, Cambridge, MA 02139; and  
†Gentest Corp., 6 Henshaw Street, Woburn, MA 01801

Communicated by Gerald N. Wogan, December 3, 1991

**ABSTRACT** Nitric oxide (NO•) is a physiological messenger formed by several cell types. Reaction with O<sub>2</sub> forms oxides that nitrosate amines at pH values near 7. We now report experiments in which NO• was added to intact human cells and to aerobic solutions of DNA, RNA, guanine, or adenine. TK6 human lymphoblastoid cells were mutated 15- to 18-fold above background levels at both the *HPRT* and *TK* gene loci. Xanthine and hypoxanthine, from deamination of guanine and adenine, respectively, were formed in all cases. NO• induced dose-responsive DNA strand breakage. Yields of xanthine ranged from nearly equal to about 80-fold higher than those of hypoxanthine. Yields of xanthine and hypoxanthine from nucleic acids were higher than those from free guanine and adenine. This was most pronounced for xanthine; 0.3 nmol/mg was formed from free guanine vs. 550 nmol/mg from calf thymus RNA. Nitric oxide added to TK6 cells produced a 40- to 50-fold increase in hypoxanthine and xanthine in cellular DNA. We believe that these results, plus the expected deaminations of cytosine to uracil and 5-methylcytosine to thymine, account for the mutagenicity of nitric oxide toward bacteria and mammalian cells.

Many types of cells produce NO• through a common biochemical pathway—i.e., the oxidation of arginine (1, 2). Although the function of this NO• is related to its role as a second messenger (3), or perhaps to targeted cytotoxicity (4), there may also be collateral reactions leading to DNA damage in neighboring cells (5, 6).

For many years it has been generally recognized that nitrite (NO<sub>2</sub><sup>-</sup>) can deaminate purines, pyrimidines, and various forms of RNA and DNA under acidic conditions; this deamination leads to mutations in prokaryotic organisms (7). These reactions proceed through a pathway in which protonation of NO<sub>2</sub><sup>-</sup> leads to formation of N<sub>2</sub>O<sub>3</sub>, an electrophilic nitrosating agent.

We proposed (8) that generation of NO• under physiological conditions in the presence of O<sub>2</sub> can reproduce the chemical and mutagenic effects of NO<sub>2</sub><sup>-</sup> under acidic conditions. Once formed, N<sub>2</sub>O<sub>3</sub> (or N<sub>2</sub>O<sub>4</sub>) can react with unprotonated amino groups in the presence of water (9). Low concentrations of NO• in the presence of O<sub>2</sub> in aqueous solutions may also lead to nitrosyl donors by other mechanisms.

We now demonstrate that, in the presence of O<sub>2</sub>, NO• can cause a variety of types of damage to DNA as well as mutations in a human lymphoblastoid cell line in culture. On the basis of these results, we propose that cytotoxicity and mutagenicity associated with the inflammatory process may result from nitrogen oxide radicals in addition to the better-known reactions of (or with) oxygen radicals.

## EXPERIMENTAL PROCEDURES

**Materials.** Xanthine, hypoxanthine, adenine, guanine, calf thymus DNA, yeast RNA, and transfer RNA (from bovine liver) were purchased from Sigma. Heat-denatured DNA was prepared by boiling calf thymus DNA solution (2.0 mg/ml) for 15 min and quickly chilling it in an ice bath. [1,3-<sup>15</sup>N<sub>2</sub>]Xanthine was purchased from Cambridge Isotope Laboratories (Cambridge, MA), and *N*-methyl-*N*-(trimethylsilyl)trifluoroacetamide was purchased from Pierce. Nitric oxide (NO•) was supplied by Matheson or Aldrich. HPLC grade ammonium acetate and organic solvents were from Fisher Scientific. [<sup>14</sup>C]Thymidine (57 mCi/mmol; 1 Ci = 37 GBq) was purchased from ICN.

**Mutagenicity Assays.** Mutagenicity assays were conducted in TK6 human lymphoblasts (10) as described (11). Treatment with nitric oxide was as follows: 100-ml cultures containing 4 × 10<sup>5</sup> cells per ml of RPMI 1640 medium supplemented with 10% horse serum were contained in 500-ml glass Erlenmeyer flasks. The mouths of the flasks were covered with sterile Parafilm. Nitric oxide was introduced directly into the medium with a sterile stainless steel cannula. The nitric oxide volume was controlled by delivery with a syringe that had been purged with nitrogen to remove oxygen. Nitric oxide was introduced into the culture at ≈1 ml/sec, and the vessel was capped and shaken. Cultures were incubated for 1 h at 37°C and then centrifuged at 1000 × *g* for 5 min. The cell pellets were resuspended in 100 ml of the medium and cultured for 6 days to allow phenotypic expression. The expressed cultures were plated in the presence of trifluorothymidine (three 96-well plates at 30,000 cells per well), in the presence of 6-thioguanine (three 96-well plates at 30,000 cells per well), or in the absence of selective conditions (two 96-well plates at 2 cells per well). After a 12-day incubation, plates were scored for the presence of a colony in each well. Nitric oxide mutagenicity was examined in two independent experiments with a total of six replicate cultures for each treatment. The positive control was 140 ng of 4-nitroquinoline-*N*-oxide per ml. The results of the two independent experiments were pooled and analyzed according to standard methods (11).

**DNA Strand Breaks.** DNA strand breaks in the TK6 cells were detected through a modified version of the DNA precipitation assay described by Olive (12). Cells in RPMI 1640 medium supplemented with 10% horse serum were radiolabeled for 20 h with 0.02 μCi of [<sup>14</sup>C]thymidine per ml in 5% CO<sub>2</sub> at 37°C followed by incubation for 2 h in fresh serum-free medium. Four milliliters of the radiolabeled TK6 cells (5 × 10<sup>5</sup> cells per ml) was placed in a 6-ml vial. Dose-response experiments were done by introducing various concentrations (0.125, 0.25, and 0.5 ml/ml)<sup>§</sup> of nitric oxide through the rubber septum into the medium. The

Abbreviation: TMS, trimethylsilyl.

<sup>‡</sup>To whom reprint requests should be addressed.

<sup>§</sup>At STP 0.1 ml of NO• equals 2.2 μmol; this was checked by analysis of nitrate and nitrite in the culture medium.

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. §1734 solely to indicate this fact.

sample was mixed and incubated at 37°C for 1, 4, and 24 h. After incubation, cells (100  $\mu$ l) were lysed in plastic tubes at 0°C for 1 min in 0.5 ml of solution containing 2% SDS, 10 mM EDTA, and 10 mM Tris to which 1 ml of 0.05 M NaOH was added just prior to lysis. KCl (0.5 ml, 0.12 M) was gently added, and the tubes were capped and placed in a water bath at 65°C for 10 min, cooled on ice for 5 min, and centrifuged for 10 min at 3500  $\times$  g. The supernatant was decanted into a liquid scintillation vial containing 1 ml of 0.05 M HCl. The pellet was resuspended twice in 1 ml of 65°C water, vortexed, and poured into a scintillation vial for determination of radioactivity by liquid scintillation counting. The percentage of DNA damage was expressed as a ratio of radioactivity in the supernatant to the sum of the radioactivities of the supernatant and the pellet multiplied by 100. Our historical background for DNA single-strand breaks by this method is 13.8%  $\pm$  4.9% (range = 3.2–24.9%; 95% confidence interval = 12.6–15.0%;  $n$  = 67). Experiments in which the background level exceeded 25% were rejected. A statistically different result from background due to treatment requires DNA damage to exceed the 95% upper confidence level of both the historical data and the concurrent control ( $P$  < 0.05).

**In Vitro Modification of Nucleic Acids and Bases.** Calf thymus DNA, yeast RNA, and transfer RNA (0.5 mg of each) and adenine and guanine (300 nmol of each) were placed individually in 1.5-ml Eppendorf tubes and dissolved in 0.5 ml of sodium phosphate buffer (50 mM, pH 7.5). The tubes were covered with rubber caps, and nitric oxide was introduced from a syringe through the rubber cap (into the liquid). The ratio of gas to solution was 0.5 ml of NO $\cdot$  per ml. The samples were vortexed for 1 min and then shaken gently in a Gyrotory water bath (New Brunswick, NJ) for 3 h at room temperature. The DNA was precipitated by addition of two volumes of cold (–30°C) ethanol, and the pellet and supernatant were separated by centrifugation. The samples were dried under vacuum and stored.

**Depurination.** DNA depurination was by mild acid hydrolysis. The dried DNA residue was dissolved in 0.25 ml of water and 0.25 ml of 0.2 M HCl, and 2 nmol of [ $^{15}$ N $_2$ ]xanthine (20  $\mu$ l of 0.1 nmol/ $\mu$ l) was added. The mixture was heated for 30 min at 80°C. After cooling, the acid hydrolysate was analyzed by HPLC.

**HPLC Analyses.** HPLC analyses were performed with a Hewlett-Packard 300 Chem Station. UV detection was by a Hewlett-Packard 1040 diode array detector over the range of 200–400 nm. Spectra of peaks detected at the monitoring wavelength were stored for later analysis. A stepwise gradient of solvent A (30 mM ammonium acetate, pH 3.2/methanol/acetonitrile; 200:5:5, vol/vol) and solvent B (double-distilled water) at a flow rate of 1.5 ml/min on a Partisil 10 strong cation-exchange (SCX) 25  $\times$  0.46 cm column was used (Whatman). The acid hydrolysate (500  $\mu$ l) was injected into the HPLC column, and the effluent containing xanthine and hypoxanthine eluting between 2.90 and 5.0 min was collected into a silanized vial, dried in a Speed-Vac, and kept for GC-MS analysis. Adenine and guanine were also quantitated by HPLC by comparing the peak area of the unknown with an external standard run under the same conditions with UV set at 254 nm.

**GC-MS Analysis of Xanthine and Hypoxanthine.** The dried residue of xanthine and hypoxanthine collected earlier was neutralized with 150  $\mu$ l of a solvent mixture (acetonitrile/methanol/ammonium hydroxide; 90:5:5, vol/vol) and dried under vacuum. The residue was dissolved in acetonitrile (100  $\mu$ l) and pyridine (20  $\mu$ l). Trimethylsilyl (TMS) derivatives were made by addition of *N*-methyl-*N*-(trimethylsilyl)trifluoroacetamide (80  $\mu$ l) and heating at 100°C for 1 h. Xanthine and hypoxanthine are completely converted to the tri-TMS and di-TMS derivatives, respectively.

The derivatized standards and samples were analyzed on a Hewlett-Packard model 5987 gas chromatograph mass spectrometer operated in the electron ionization mode. The electron energy was 70 eV. The source temperature was 210°C. Dwell times were 200 msec per ion. The columns were fused-silica capillary, either a 12 m  $\times$  0.2 mm HP-1 (Hewlett-Packard) or a 15 m  $\times$  0.25 mm DB-5 (J & W Scientific, Rancho Cordova, CA). The injection port and transfer lines were at 240°C. The carrier gas was ultra-high-purity helium (Medtech, Medford, MA), with a flow rate of about 2 ml/min. Samples were injected in the splitless mode with an initial oven temperature of 150°C for 2 min, followed by a temperature program to 220°C at 10°C/min, then to 280°C at 25°C/min, with a final hold at 280°C for 2 min. The electron ionization mass spectra are characterized by a base peak at  $M$  – 15. When analyzed at similar concentrations, the nucleic acid bases and the compounds under study were well resolved on a 12-m HP-1 fused-silica capillary column. The levels of the compounds of interest in the DNA hydrolysate were small compared to the levels of adenine and guanine, so it is necessary to perform HPLC clean-up prior to GC-MS analysis. Adenine and guanine can be quantitated simultaneously in the same HPLC separation.

Quantitative analysis was carried out by monitoring the  $M$  – 15 ion of the TMS-derivatized [ $^{15}$ N $_2$ ]xanthine at  $m/z$  355 as internal standard; for xanthine and hypoxanthine, the corresponding ions at  $m/z$  353 and  $m/z$  265 were used.

## RESULTS

**Nitric Oxide Cytotoxicity and Mutagenicity.** Nitric oxide was tested for mutagenicity at concentrations of 0.125, 0.25, and 0.375 ml of nitric oxide gas per ml of culture medium for 1 h after direct addition of nitric oxide to the medium. This treatment induced a substantial concentration-dependent decrease in relative survival and concentration-dependent increase in mutant fraction at both the *HPRT* and *TK* loci in TK6 human lymphoblasts (Fig. 1).

The concentration dependence was approximately linear; the highest concentration tested, 0.375 ml of nitric oxide per ml of culture medium, induced a 15- to 18-fold increase in the mutant fraction over the concurrent negative control values at both loci.

Nitric oxide was cytotoxic at all concentrations tested. The relative survival at the highest concentration was 0.12. The fold increase in the mutant fraction was, at all concentrations, greater than 1/(relative survival). Therefore systemic bias (i.e., preferential survival of mutant cells) could not have caused the response. This is supported by reconstruction experiments, which found no systematic bias in the TK6 system (10).

The addition of nitric oxide resulted in a modest decrease in pH. Cultures exposed to 0.125 and 0.25 ml of nitric oxide per ml had a pH in the range of pH 6.6–7.0. Cultures exposed to 0.375 ml of nitric oxide per ml had a pH of 6.1. In some *in vitro* mutagenicity assays, exposure to low pH is mutagenic (15, 16). We believe that the pH drop is not a factor in the mutagenicity of nitric oxide because a mutagenic response was observed at normal pH. In addition, the concentration of HNO $_2$  ( $pK_a$  = 3.4) is negligible above pH 6 (i.e., the effects could not have resulted from protonation of nitrite).

This conclusion is supported by our observation that nitric oxide was nontoxic and nonmutagenic to TK6 cells when the gas was injected into the culture headspace; a comparable decrease in pH had no significant effect on the mutant fraction or relative survival.

**DNA Strand Breaks.** Under conditions comparable to those used for the mutation experiments, NO $\cdot$  elicited both dose- and time-dependent DNA single-strand breaks in treated TK6 cells (Fig. 2). One hour after treatment, there was no

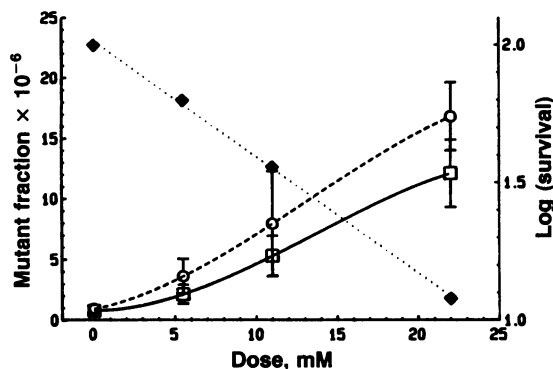


FIG. 1. Relative survival ( $\blacklozenge$ ) was calculated based on analysis of cell growth after nitric oxide treatment (13, 14). Values are plotted as means. Mutant fraction values are plotted as the mean and standard deviation of six replicate observations.  $\circ$ , values at the *TK* locus;  $\square$ , values at the *HPRT* locus. The historical negative control data base for these gene loci in TK6 cells contained 70 observations with a mean mutant fraction of  $1.41 \times 10^{-6}$  and standard deviation of  $0.7 \times 10^{-6}$  for the *HPRT* locus and 309 observations with a mean mutant fraction of  $1.35 \times 10^{-6}$  and a standard deviation of  $0.76 \times 10^{-6}$  for the *TK* locus. Exposure to 0.25 and 0.375 ml of nitric oxide per ml of culture resulted in a statistically significant increase in the mutant fraction when compared to the concurrent negative control (Dunnett's *t* test, 95% confidence level) and the historical negative control data base (9). The positive control, 140 ng of 4-nitroquinoline-*N*-oxide per ml, induced mutant fractions of  $3.7 \times 10^{-5}$  and  $2.5 \times 10^{-5}$  at the *TK* and *HPRT* loci, respectively.

significant difference in the amount of DNA damage in untreated cells and cells that were treated with 0.125 and 0.25 ml of  $\text{NO}^\bullet$  per ml, although the high dose of  $\text{NO}^\bullet$  (0.5 ml/ml) caused DNA damage that was significantly higher than the background level. Four hours after exposure, DNA damage in all treated cultures was significantly higher than background levels. The highest dose again caused the highest level of DNA damage. Twenty-four hours after treatment, no difference in DNA damage was noted between the untreated sample and the sample dosed at 0.125 ml/ml, although the two highest doses of  $\text{NO}^\bullet$  treatment caused extensive DNA damage.

The nonlinearity observed in these experiments suggested that DNA damage caused by  $\text{NO}^\bullet$  treatment might be repaired by an inducible enzyme system. To test this possibility, TK6 cells were first treated with 0.125 and 0.250 ml of  $\text{NO}^\bullet$  per ml. One or 20 h later, both cultures were treated with 0.5 ml of  $\text{NO}^\bullet$  per ml, and DNA damage was assessed after a total exposure of 4 or 24 h, respectively. Both treated cultures contained over 70% damaged DNA after 4 and 24 h of

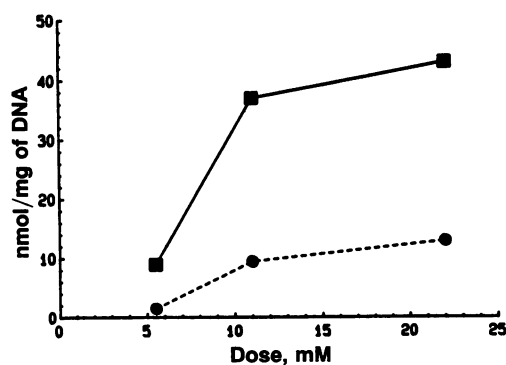


FIG. 2. Calf thymus DNA was treated with nitric oxide and then deproteinized by mild acid hydrolysis. The resulting xanthine ( $\blacksquare$ ) and hypoxanthine ( $\bullet$ ) were quantitated by GC-MS using [ $^{15}\text{N}_2$ ]xanthine as the internal standard. Statistically significant dose-related increases were observed with both compounds.

treatment. The similarity in the levels of DNA damage with and without  $\text{NO}^\bullet$  pretreatment suggested that DNA damage caused by  $\text{NO}^\bullet$  treatment is not repaired by an inducible enzyme system.

We also investigated whether DNA damage caused by successive  $\text{NO}^\bullet$  treatments was additive. Two cultures of TK6 cells were dosed with 0.5 ml of  $\text{NO}^\bullet$  per ml, either in one dose or in four hourly doses of 0.125 ml/ml. The relationship between dose and DNA damage is complex, and it is probable that the delivered dose is greater with multiple small doses than with a single larger dose of equivalent volume. Although both cell cultures exhibited significant DNA damage, the culture that had been dosed in four treatments experienced the higher level of DNA damage. These experiments suggest that the treatments are approximately additive and that in this cell line neither induction nor repair of  $\text{NO}^\bullet$ -induced DNA damage takes place.

**Deamination of Purine Bases.** The deamination of purines from various nucleic acid substances after treatment with nitric oxide is summarized in Table 1. Neither xanthine nor hypoxanthine was detected in either the pellet or the supernatant of the control samples. Deamination of free adenine occurs in higher amounts than that of free guanine. However, the level of deamination of nucleic acids occurring at guanine bases was much higher than that at adenine bases. The deamination of guanine in yeast RNA was almost complete, with about 78% conversion to xanthine. Calf thymus DNA exhibited a monotonic dose-response to  $\text{NO}^\bullet$  over the entire range of doses (Fig. 3).

Deaminated bases were also found in DNA isolated from cultured cells treated with nitric oxide. About  $10^8$  cells were used in DNA isolation from TK6 cells. The DNA measured by UV spectroscopy was 670  $\mu\text{g}$  and 625  $\mu\text{g}$  for the control and the  $\text{NO}^\bullet$ -treated cells, respectively. From the DNA of the treated cells, hypoxanthine was found to be 5.3 nmol/mg of DNA, and xanthine was 2.8 nmol/mg of DNA. These are 39 and 47 times higher than the hypoxanthine and xanthine in the control DNA. Only the DNA pellet was analyzed in this experiment, and it is presumed that additional xanthine and hypoxanthine were present in the cell supernatant.

## DISCUSSION

Nitric oxide induced a concentration-dependent increase in the mutant fraction over the dose range of 0.125–0.375 ml of nitric oxide gas per ml of culture medium. The mutant fractions at both gene loci increased to 15- to 18-fold over the concurrent negative controls. The results at both the *TK* and *HPRT* loci were qualitatively and quantitatively similar, which is consistent with previous observations (10).

The nitric oxide concentrations that induced a significant response were relatively high ( $>5$  mM). For comparison, several direct-acting, electrophilic mutagens, such as nitrosomethylurea, ICR-191, and 4-nitroquinoline-*N*-oxide, are active in TK6 cells at the 0.1–10  $\mu\text{M}$  range (10). Millimolar concentrations of other mutagens, such as butyl methane-

Table 1. Nitric oxide deamination of purine bases from various sources

Sample	Hypoxanthine, nmol/mg of nucleic acids	Xanthine, nmol/mg of nucleic acids
Adenine	0.6	—
Guanine	—	0.3
Calf thymus DNA	8.4	39.3
Yeast RNA	7.2	549*
Bovine liver tRNA	2.6	32.8

Samples were treated with nitric oxide at 22  $\mu\text{mol/ml}$ .

\*Eighty percent of the total guanine.

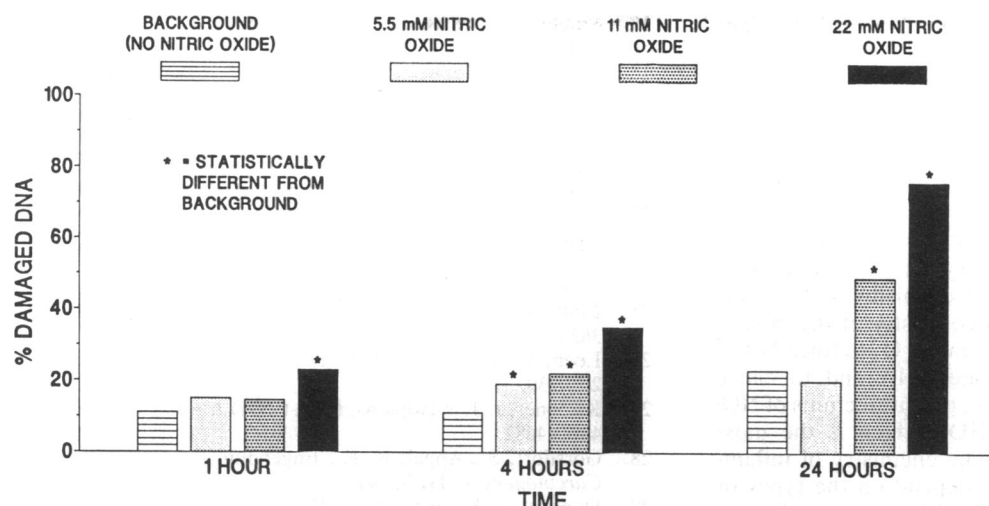


FIG. 3. TK6 cells were radio-labeled with [ $^{14}\text{C}$ ]thymidine, treated with nitric oxide, and then analyzed for DNA single-strand breaks by a precipitation assay (12). DNA damage is expressed as the percentage of radioactivity in the pellet and the supernatant following precipitation with respect to the total radioactivity in the pellet and the supernatant. Both dose-related and time-related increases in strand breakage were observed. The method of statistical analysis is described in the text.

sulfonate, are sometimes needed to observe statistically significant mutagenic responses. However, the method of delivery of  $\text{NO}$  used in these experiments is relatively inefficient, and it is probable that a great deal escapes into the gas phase without forming  $\text{N}_2\text{O}_3$  in solution. In contrast, macrophages may produce pmol per cell per h over days (17, 18) so that neighboring cells would be exposed to a constant barrage of  $\text{N}_2\text{O}_3$ . Although earlier workers had demonstrated the bacterial mutagenicity of methyl nitrite (19) and malignant transformation of 3T3 cells by sodium nitrite (20), we believe this is the first report of mutagenicity by  $\text{NO}$  in mammalian cells under normal culture conditions.

The mechanisms of mutagenesis of cells by  $\text{NO}$  are uncertain, but our results suggest some alternative non-mutually exclusive hypotheses. The ability of  $\text{N}_2\text{O}_3$  to deaminate aromatic amines via their aryl diazonium ions is well known as is the mechanism of acid-catalyzed nitrite transformation in nucleic acids (7). The rates and products of these reactions for purine and pyrimidine bases and nucleosides and nucleic acids have been established by the elegant studies of Shapiro and Pohl (21). It is clear from these earlier studies and the present work that relative rates of deamination depend on both the individual base and the nucleic acid structure, but, in DNA, guanine is relatively more reactive than adenine, whereas cytosine and adenine are comparable. This is in marked contrast to the thermal hydrolytic deamination reactions in which cytosine > adenine >> guanine (22, 23). Cytosine deamination in double-stranded DNA in *Escherichia coli* is extremely slow, with a half-life of about 30,000 years, whereas that for single-stranded DNA is 200 years (24). Therefore, catalyzed deamination may add greatly to the burden of existing DNA repair systems.

In addition to mispairing of deaminated bases, the instability of hypoxanthine and xanthine in DNA leads to depurination and subsequent strand breakage (25). Both of these phenomena have been observed in calf thymus DNA and in intact cells in our experiments and potentially contribute to both cytotoxicity and mutagenicity (26). A third possibility, not explored here, is the formation of intrastrand cross-links via attack of the aryl diazonium ion of one purine on the free amino group of a second purine in the matching strand (7, 27). This type of cross-linking is presumably more likely to result in toxicity than in mutagenesis. Earlier work had shown that single-strand breaks could be caused by  $\text{NO}_2$  but not  $\text{NO}$  in the absence of  $\text{O}_2$ , supporting the notion that these breaks occur as a result of nitrosative deamination (28).

Our observation that  $\text{NO}$  deaminates guanine faster than other bases suggests an important difference from the inescapable thermal hydrolytic mechanism. Deamination products of cytosine and adenine may be repaired (29, 30), but the

extreme instability of xanthine in DNA (31) leads to its rapid depurination (32). The rate of deamination of guanine may normally be so slow, and the half-life of xanthine may be so short that no excision repair system evolved.

The mutations that might arise from nitrosative deamination are summarized in Table 2. Based on relative rates of deamination of the different bases in DNA, we would predict that G-C  $\rightarrow$  A-T transitions would predominate in molecular mechanisms of mutagenesis in cells exposed to  $\text{NO}$ . This type of mutation has been shown to result from the exposure of *E. coli* to  $\text{NO}$  (36). This is supported by the observation that tetranitromethane, a nitrosating agent, yields transformed K-ras genes with predominantly a G-C  $\rightarrow$  A-T transition in lung tumors of treated rats and mice (37). In addition, an earlier analysis of the nitrous acid mutagenesis literature also concludes that in viruses and yeast, transitions, particularly G-C  $\rightarrow$  A-T, best explain the amino acid substitutions found in expressed genes (38). These are not unique transformations because similar selectivity could also arise via exposure to other chemicals.

An analysis of p53 mutations in human cancers (39) demonstrates that in the most prevalent tumor sites two types of conversions dominate. In colon cancer, transitions at CpG dinucleotide mutational hot spots could arise via methylation at cytosine followed by deamination to thymine induced by  $\text{NO}$  released as part of the inflammatory process. This hypothesis is supported by the observation that nitrite accumulates in the lower gastrointestinal tract under pathological circumstances (40). On the other hand, transversions in p53 of G-C  $\rightarrow$  T-A are most frequently seen in liver and lung cancers, and these could be related to  $\text{NO}$ -induced depurination (26). In the case of hepatocellular carcinoma, it has been shown that hepatitis B virus infection in woodchucks leads to increased urinary nitrate (41). Studies on one human subject with chronic active hepatitis resulting from hepatitis B virus infection also demonstrate greatly elevated urinary nitrate output (C. Leaf and S.R.T., unpublished results). Elevated urinary nitrate has also been found in humans infected with liver flukes at elevated risk for cholangiocarcinoma (42) and in populations infected with *Schistosomiasis*

Table 2. Types of mutations that potentially arise from deamination of DNA bases

Conversion	Type of mutation	Ref.
5-Methylcytosine $\rightarrow$ thymine	G-C $\rightarrow$ A-T	33
Cytosine $\rightarrow$ uracil	G-C $\rightarrow$ A-T	34
Adenine $\rightarrow$ hypoxanthine	A-T $\rightarrow$ G-C	35
Guanine $\rightarrow$ xanthine $\rightarrow$ apurinic site	G-C $\rightarrow$ T-A	26
Adenine $\rightarrow$ hypoxanthine $\rightarrow$ apurinic site	A-T $\rightarrow$ T-A	26

*mansoni* (43). Since the nature of mutations in key genes (protooncogenes and/or tumor suppressor genes) in the last two tumor sites is unknown, these might be important experiments to test the generality of our hypothesis that nitrosative deamination is an important mechanism of mutagenesis leading to some human cancers.

Inflammation attracts many types of cells to the affected tissue or organ. These cells have traditionally been viewed as important sources of oxygen radicals, which in turn are implicated in mechanisms of cell injury (44). The addition of NO $\cdot$  to the armament of defense mechanisms adds a new dimension to the chemistry and biochemistry of the inflammatory process. NO $\cdot$ , via its reaction with O $_2$  to form N $_2$ O $_3$ , has been shown to directly damage DNA and to cause mutations in human cells. In addition, the interactions of NO $\cdot$  and O $_2^{\cdot-}$  (45, 46) may generate HO $\cdot$ , which is the most damaging of the oxygen radicals. The chemistry of inflammation in any tissue will therefore depend on the types of leukocytes migrating to that tissue. This pattern will vary with both time and agent. Analysis of this problem *in situ* in specific organs will require specific probes and methods of dosimetry for all types of implicated reactants and their products related to cell injury.

This work was supported by National Institutes of Health Grants CA26731 and ES02109. D.B. was supported by a National Institutes of Health Fellowship (MARC no. GM12248).

- Iyengar, R., Stuehr, D. J. & Marletta, M. (1987) *Proc. Natl. Acad. Sci. USA* **84**, 6369–6373.
- Marletta, M. A., Yoon, P. S., Iyengar, R., Leaf, C. D. & Wishnok, J. S. (1988) *Biochemistry* **27**, 8706–8711.
- Moncada, S., Palmer, R. M. J. & Higgs, A. (1991) *Pharmacol. Rev.* **43**, 109–142.
- Hibbs, J. B., Jr., Taintor, R. R. & Vavrin, Z. (1987) *Science* **235**, 473–486.
- Nguyen, T. T., Brunson, D. C., Wishnok, J. S., Tannenbaum, S. R. & Crespi, C. L. (1991) *Proc. Am. Assoc. Cancer Res.* **32**, 644 (abstr.).
- Wink, D. A., Kasprzak, K. S., Maragos, C. M., Misra, M., Dunams, T. M., Andrews, A. W. & Keefer, L. K. (1991) *Proc. Am. Assoc. Cancer Res.* **32**, 673 (abstr.).
- Singer, B. & Grunberger, D. eds. (1983) in *Molecular Biology of Mutagens and Carcinogens* (Plenum, NY), pp. 47–48.
- Nguyen, T. T., Wishnok, J. S., Tannenbaum, S. R. & Crespi, C. L. (1991) *Proc. Am. Assoc. Cancer Res.* **32**, 108 (abstr.).
- Challis, B. C. & Kyrteopoulos, S. A. (1978) *J. Chem. Soc. Perkin Trans. 2* **2**(12), 1296–1302.
- Liber, H. L. & Thilly, W. G. (1982) *Mutat. Res.* **94**, 467–485.
- Penman, B. W. & Crespi, C. L. (1987) *Environ. Mol. Mutagenesis* **10**, 35–60.
- Olive, P. L. (1988) *Environ. Mol. Mut.* **11**, 487–495.
- Crespi, C. L., Seixas, G. M., Turner, T. R., Ryan, C. G. & Penman, B. W. (1985) *Mutat. Res.* **142**, 133–140.
- DeLuca, J. G., Kaden, D. A., Komives, E. A. & Thilly, W. G. (1984) *Mutat. Res.* **128**, 47–57.
- Brusick, D. J. (1987) *Mutat. Res.* **189**, 1–6.
- Cifone, M. A., Myhr, B. A., Eiche, A. & Bolcsfoldi, G. (1987) *Mutat. Res.* **189**, 39–46.
- Stuehr, D. J. & Marletta, M. A. (1987) *Cancer Res.* **47**, 5590–5594.
- Miwa, M., Stuehr, D. J., Marletta, M. A., Wishnok, J. S. & Tannenbaum, S. R. (1987) *Carcinogenesis* **8**, 955–958.
- Tornqvist, M., Rannug, U., Jonsson, A. & Ehrenberg, L. (1983) *Mutat. Res.* **17**, 47–54.
- Tsuda, H. & Hasegawa, M. (1990) *Carcinogenesis* **11**, 595–597.
- Shapiro, H. S. & Pohl, S. H. (1968) *Biochemistry* **7**, 448–455.
- Shapiro, R. & Klein, R. S. (1966) *Biochemistry* **5**, 2358–2362.
- Lindahl, T. & Nyberg, B. (1974) *Biochemistry* **13**, 3405–3410.
- Frederico, L. A., Kunkel, T. A. & Shaw, B. R. (1990) *Biochemistry* **29**, 2532–2537.
- Lindahl, T. & Andersson, A. (1972) *Biochemistry* **11**, 3618–3623.
- Loeb, L. A. & Preston, B. D. (1986) *Annu. Rev. Genet.* **20**, 201–230.
- Kirchner, J. J. & Hopkins, P. B. (1991) *J. Am. Chem. Soc.* **113**, 4681–4682.
- Gorsdorf, S., Appel, K. E., Engholm, C. & Obe, G. (1990) *Carcinogenesis* **11**, 37–41.
- Duncan, B. K., Rockstroh, P. A. & Warner, H. R. (1978) *J. Bacteriol.* **134**, 1039–1045.
- Duncan, J., Hamilton, L. & Friedberg, E. C. (1976) *J. Virol.* **19**, 338–345.
- Moschel, R. C. & Keefer, K. K. (1989) *Tetrahedron Lett.* **30**, 1467–1468.
- Shapiro, H. S. & Chargaff, E. (1966) *Biochemistry* **5**, 3012–3018.
- Coulondre, C., Miller, J. H., Farabaugh, P. J. & Gilbert, W. (1978) *Nature (London)* **274**, 775–780.
- Duncan, B. K. & Miller, J. (1980) *Nature (London)* **287**, 560–561.
- Karran, P. & Lindahl, T. (1980) *Biochemistry* **19**, 6005–6011.
- Wink, D. A., Kasprzak, K. S., Maragos, C. M., Elespuru, R. K., Misra, M., Dunams, T. M., Cebula, T. A., Koch, W. H., Andrews, A. W., Allen, J. S. & Keefer, L. K. (1991) *Science* **254**, 1001.
- Stowers, S. J., Glover, P. L., Reynolds, S. H., Boone, L. R., Maronpot, R. R. & Anderson, M. W. (1987) *Cancer Res.* **47**, 3212–3219.
- Zimmerman, F. K. (1977) *Mutat. Res.* **39**, 127–148.
- Hollstein, M., Sidransky, D., Vogelstein, B. & Harris, C. C. (1991) *Science* **253**, 49–53.
- Roediger, W. E. W., Lawson, M. J. & Radcliffe, B. C. (1990) *Dis. Colon Rectum* **33**, 1034–1036.
- Liu, R. H., Baldwin, B., Tennant, B. C. & Hotchkiss, J. H. (1991) *Cancer Res.* **51**, 3925–3929.
- Srianujata, S., Tonbuth, S., Bunyaratvej, S., Valyasevi, A., Promvanit, N. & Chaivatsagul, W. (1987) in *The Relevance of N-Nitroso Compounds in Human Cancer: Exposures and Mechanism*, eds. Bartsch, H., O'Neill, I. K. & Shulte-Herman, R. (Int. Agency Res. Cancer, Lyon, France), Int. Agency Res. Cancer Publ. no. 84, pp. 544–546.
- Tricker, A. R., Mostafa, M. W., Spiegelhalter, B. & Preussmann, R. (1989) *Carcinogenesis* **10**, 547–552.
- Fridovich, I. (1983) *Annu. Rev. Pharmacol. Toxicol.* **23**, 239–271.
- Blough, N. V. & Zafiriou, O. C. (1985) *Inorg. Chem.* **24**, 3504–3505.
- Beckman, J. S., Beckman, T. W., Chen, J., Marshall, P. A. & Freeman, B. A. (1990) *Proc. Natl. Acad. Sci. USA* **87**, 1620–1624.